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TITLE: Examination of the mGluR-mTOR Pathway for the Identification of Potential Therapeutic Targets To Treat Fragile X

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14. ABSTRACT

Fragile X Syndrome (FXS) is a single gene disorder caused by loss of *FMR1* gene function. This disease leads to cognitive impairment and is the most common genetic cause of autism, accounting for 2-6% of all diagnosed cases (Hagerman et al 2008). In previous studies of a *Drosophila* model for FXS, we identified pharmacological treatments that rescued phenotypes relevant to this syndrome such as social, neuroanatomical and cognitive deficits (McBride et al., 2005; Choi et al., 2010). These results have been translated to the mouse model of FXS leading to the impetus to initiate clinical trials with Fragile X patients (Yan et al., 2005; Dolen et al., 2007; de Vrij et al., 2008; Choi et al., 2011). The fact that clinical trials of two distinct compounds identified in flies and tested in mice have reported some level of efficacy highlights the relevance of *Drosophila* and mouse-based disease modeling to identify potential treatments for developmental brain disorders and other diseases (Berry-Kravis et al., 2008; Berry-Kravis et al., 2009;

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Introduction

Fragile X syndrome is the leading cause of intellectual disability resulting from a single gene mutation. Previously, we characterized social and cognitive impairments in a *Drosophila* model of Fragile X syndrome and demonstrated that these impairments were rescued by treatment with metabotropic glutamate receptor (mGluR) antagonists or lithium. In the mouse model of Fragile X a well-characterized phenotype is enhanced mGluR-dependent long-term depression (LTD) at Schaffer collateral to CA1 pyramidal synapses of the hippocampus. Last year we have reported the use of PDE-4 inhibitors in rescuing social, and memory phenotypes in the mouse as well as the enhanced-LTD phenotype observed in the *Fmr1* mouse KO. Last year we also reported the finding that metformin treatment also rescues the memory phenotype in the fly model of Fragile X. In this year we have focused on metformin treatment in the fly model and prepared to perform metformin treatment in the mouse to determine if it can also rescue memory and other phenotypes in the mouse model.

Metformin is an important drug to test in the fly and mouse models. One of the known targets of metformin is AMPK. By activating AMPK, it reduces the activity of the TORC1(mTOR) pathway (see figure 1 below) Most importantly, metformin is an FDA approved drug that has a very safe and long clinical history. It is commonly used to treat type II diabetes in humans and has recently been used to treat weight gain in patients treated with anti-psychotics. It is safe enough to prescribe to children and is now routinely prescribed to children as young as 5 years of age. If metformin is effective in the fly and mouse model, clinical trials with Fragile X patients would clearly be warranted.

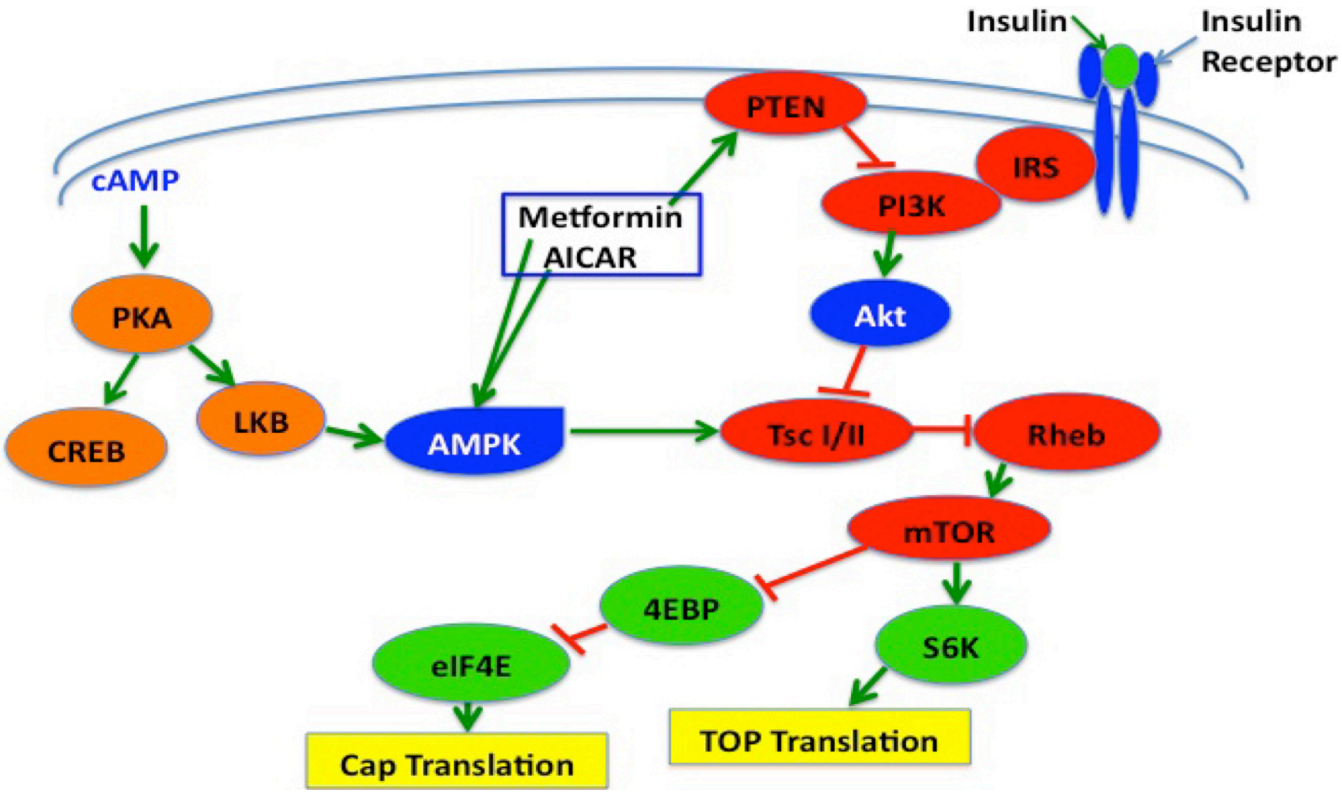


Figure 1. Insulin, mTOR signaling pathway the activity of metformin. Metformin has two activities that act to reduce mTOR signaling activity. First it acts to activate AMPK which increases the activity of the TSCI/II complex and represses Rheb activity more, thus activating mTOR less. Metformin also activates the transcription of PTEN, which results in increased repression of PI3K and less activation of Akt and thus less repression of TscI/II and again adding to the repression of Rheb and thus less activation of mTOR.

To study the efficacy of metformin in more detail, we have tested the effect of treating *dfmr1* mutants during development, during adulthood or both and tested for short-term memory as well as for rescue of circadian behavior. We found that we could not rescue the circadian defect (**Fig 2**).

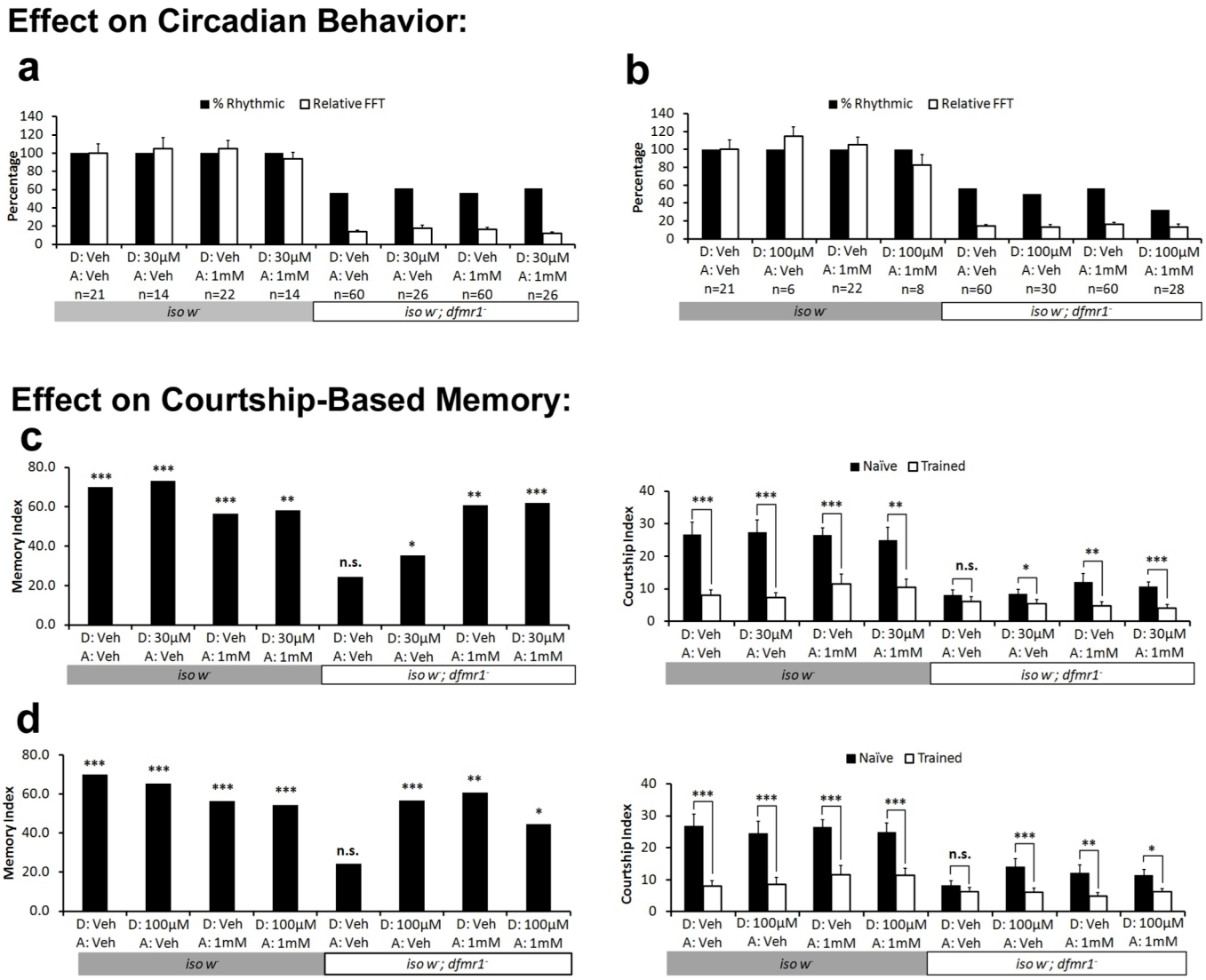


Figure 2. Effect of developmental and adulthood metformin treatment on circadian behavior and courtship-based memory. a-b, The circadian behavior of flies raised on **a,** 30µM or **b,** 100µM metformin and moved to 1mM metformin or vehicle control food within 24 hour of eclosion was examined. Metformin treatment did not improve the rhythmicity of *dfmr1* mutants. **c-d,** Flies raised on **c,** 30µM metformin or **d,** 100 µM metformin and moved to 1mM metformin or vehicle control food within 24 hours of eclosion were tested in the conditioned courtship paradigm. Treatment with either 30µM or 100µM metformin in development alone, or paired with 1mM metformin treatment in adulthood rescued STM in *dfmr1* mutant flies. Both MIs and CIs are displayed for each experiment. N ranged between 17-27.

Completion of metformin studies in the *Fmr1* KO mouse model of Fragile X Syndrome.

We demonstrated that metformin treatment in the mouse was well tolerated (**Fig 3**). We observed that the *Fmr1* KO mouse maintain a relatively normal weight profile with high dosage of metformin. Metformin did not affect rate of weight gain through development into adulthood.

We used two protocols for metformin administration through drinking water at a 0.3mg/ml concentration. Adulthood treatment consisted of 3 weeks of metformin administration starting at 9 weeks of age. Developmental treatment began from weaning through adulthood with a minimum of 9 weeks of metformin administration. Often patients are administered metformin for an extended period of time and thus, the developmental protocol for metformin administration more closely tracks possible therapeutic use.

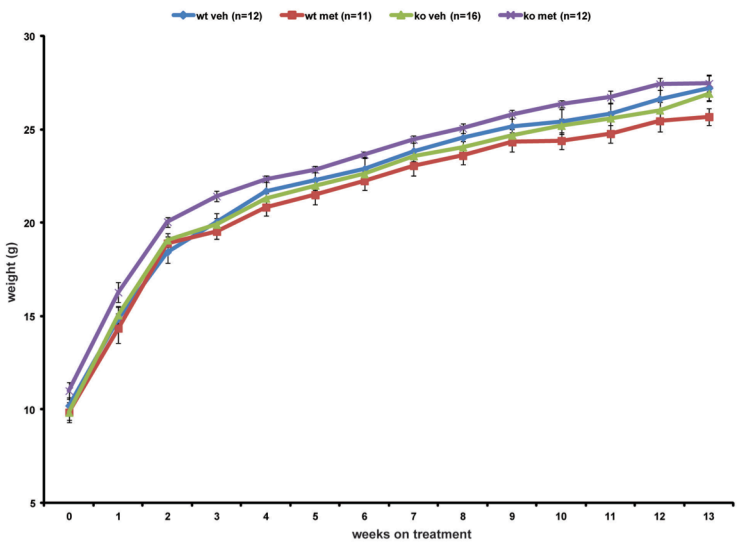


Figure 3. Effect of high dose metformin on *Fmr1* KO and control mice to determine how well the drug is tolerated. We find that mice that were given a high dose of metformin (2.0mg/ml) (a comparable dose for type II diabetes patients would be 200mg/ml) maintain normal weight and do not show any obvious negative effects of metformin treatment. They were placed on metformin at 3 weeks of age.

To examine the effects of metformin treatment on physical phenotypes in the *Fmr1* KO mice, we dissected and measured the weights of the testes in adult mice (15+ weeks of age). Macroorchidism is a well characterized physical phenotype of FXS syndrome that is recapitulated in *Fmr1* KO mice. Administration of 0.3 mg/ml metformin *ad libitum* via drinking water for 3 weeks starting at 9 weeks of age (adulthood administration) or continuously from 3 weeks of age until adulthood (developmental administration) did not result in significant change in testes size (**Fig 4**).

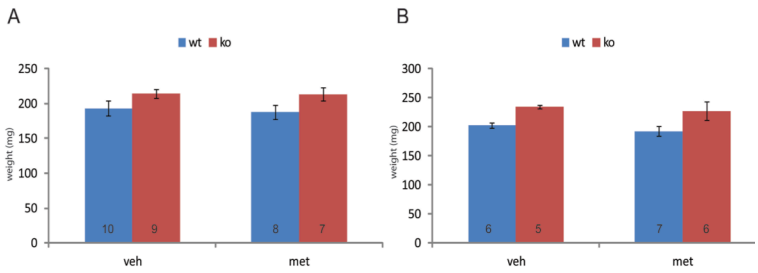


Figure 4. Effect of developmental and adulthood metformin on macroorchidism in *Fmr1* KO mice. *Fmr1* KO mice have enlarged testes compared to WT mice. Metformin treatment did not affect testes size in *Fmr1* knockout mice with adulthood (**A**) or developmental (**B**) application at 0.3mg/ml. N is indicated by the number in the bar graphs.

We had previously established conditions to perform the rotarod test that show a clear and reproducible difference between the mutants and littermate controls. We examined the effects of metformin on rotarod performance, which measures motor learning and coordination. Treatment with metformin did not significantly affect performance in the rotarod task (**Fig 5**).

As proof of principle, we examined the abundance of phosphorylated AMPK and phosphorylated mTOR in the brains of *Fmr1* KO mice and wildtype mice after metformin administration. Interestingly, we found that metformin modulates AMPK phosphorylation (data not shown) and mTOR phosphorylation only after adulthood treatment and not developmental treatment (**Fig 6**).

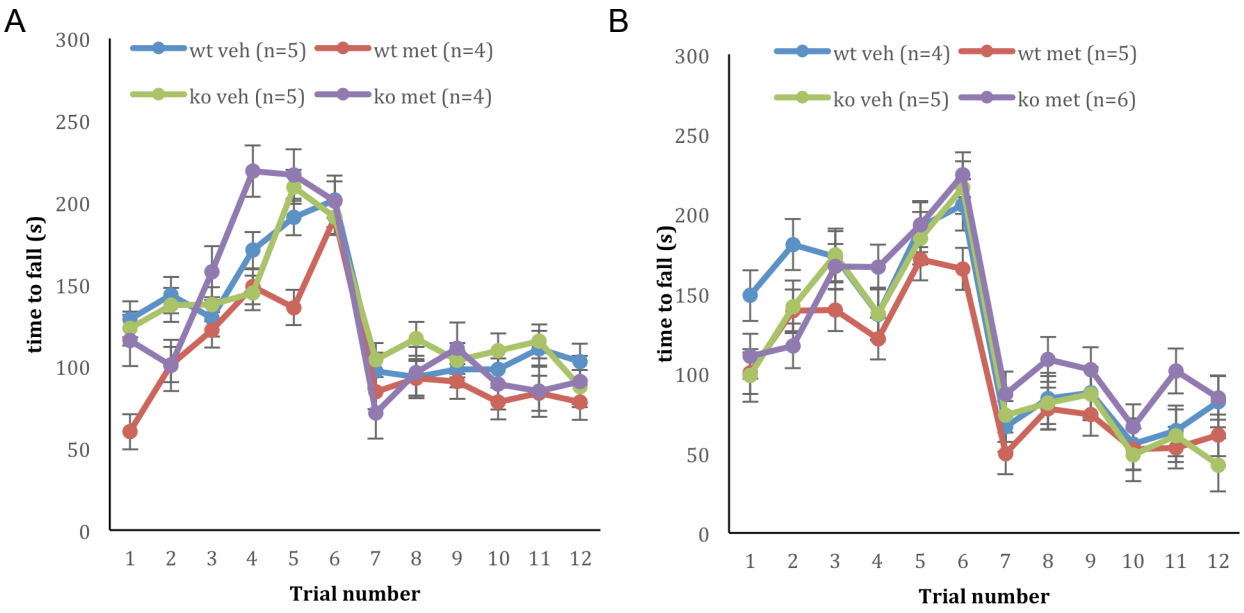


Figure 5. Effect of developmental and adulthood metformin on rotarod performance in *Fmr1* KO mice. *Fmr1* KO mice and wildtype littermate controls were trained on the rotarod task over 4 days with 3 trials per day. During the first 6 trials, the rotarod accelerated from 4 rpm to 40 rpm. During the last 6 trials, the rotarod accelerated from 8 rpm to 80 rpm. Time to fall off the rotarod was measured in seconds. Metformin treatment did not affect rotarod performance in *Fmr1* knockout mice with adulthood (**A**) or developmental (**B**) application.

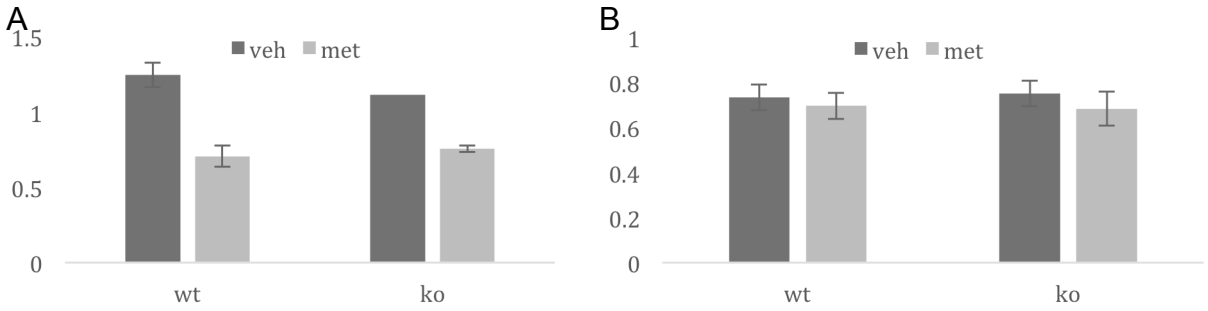


Figure 6. Effect of developmental and adulthood metformin on mTOR phosphorylation in the cerebellum of *Fmr1* KO mice. Cerebellum was dissected and processed to analyze mTOR phosphorylation in *Fmr1* KO mice and wildtype littermate controls. Metformin treatment at 0.3mg/ml significantly decreased mTOR phosphorylation with adulthood application (**A**), but not with developmental (**B**) application. N values ranged 4 to 7.

After extensive behavioral testing of *Fmr1* KO mice, we determined that few reported phenotypes could be reproduced in our hands. This includes marble burying, novel object recognition, object place memory, and reversal learning in the water Y maze (data not shown).

It has been previously reported that *Fmr1* KO mice are hypoactive compared to wildtype littermates. However, we have found hypoactivity in the during the dark and active period. Furthermore, there is a slight but significant hyperactivity during the light phase in *Fmr1* KO mice (**Fig 7**). Interestingly, preliminary data suggests that hypoactivity during the active phase can be rescued with adulthood metformin administration

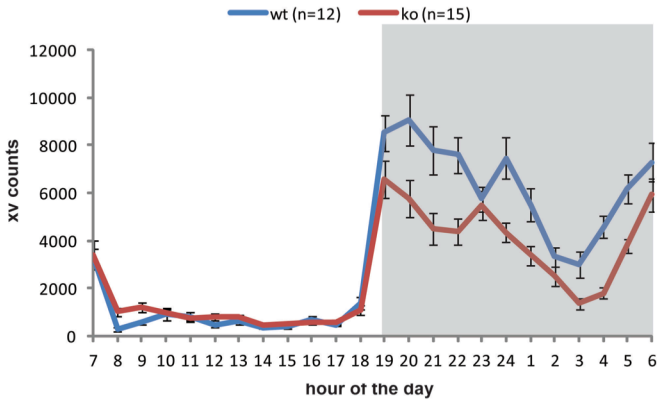


Figure 7. *Fmr1* KO mice is hypoactive in the dark period. Activity of *Fmr1* KO mice were measured using infrared beam breaks over a period of 2 weeks. Compared to wildtype littermate controls, *Fmr1* KO mice were consistently hypoactive during the dark phase.

(**Fig 8**), but not developmental metformin administration (data not shown).

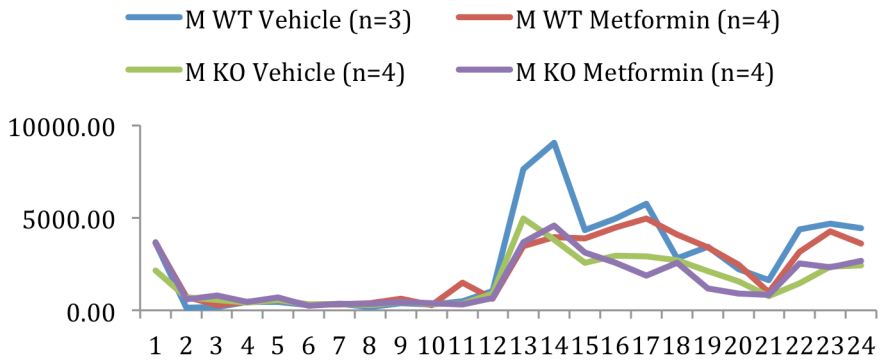


Figure 8. Effect of adulthood metformin on activity in *Fmr1* KO mice. Metformin treatment rescued the hypoactivity seen in *Fmr1* knockout mice after adulthood treatment.

Key Research Accomplishments:

Task 12c-The behavioral testing of *Fmr1* KO mice on C57Bl/6J background and the 129xBL6 background is complete. These crosses did not make the *Fmr1* phenotypes more prominent. We were able to complete all our behavioral studies using the C57BL/6J background and found a new phenotype, which we will be reporting with manuscripts in preparation.

Task ???-Metformin treatment demonstrated mixed results. Treatment over a short period of time was able to rescue some phenotypes, but not others. Chronic treatment over an extended period of time through development was unable to rescue any phenotype. This suggests that a compensatory mechanism exists to limit the impact of metformin on the mTOR pathway, which diminishes enthusiasm for its possible use in treatment of behavioral deficits in Fragile X Syndrome.

Manuscripts in preparation:

Tudor, J.C., Wei, W., Davis, E.J., Chung, C.W., Abel, T., and Jongens, T. Metformin can rescue behavioral deficits in a mouse model of Fragile-X syndrome but not with chronic use.

Tudor, J.C., Davis, E.J., Jongens, T., and Abel, T. The Fmr1 knockout mouse model behavior phenotype is fluid.

Completion of PDE4 studies in the *Drosophila* fragile X model. (items 2a, 4a and b, 5a, and 8a on Statement of Work.)

For Task 2a we have established several stocks to genetically validate the results that indicate that pharmacological inhibition of Gsk3beta activity can rescue Short-term memory, as well as to examine the impact of targeting downstream members of the mGluR signaling pathway. These stocks are currently being validated.

Task 4: In studies to examine the effects of PI3K antagonists on the *dfmr1* mutants we have found that the naïve courtship and short-term memory phenotypes can be rescued. These results have been validated genetically as shown below. Interestingly we have also been able to rescue the circadian defect displayed by the *dfmr1* mutants. The circadian phenotype however was not rescued by the pharmacological treatment. In experiments unrelated to this grant we have found that the critical period for decreasing PI3K activity with respect to circadian behavior is during the pupal period, a time in development that we cannot administer pharmacological agents, easily.

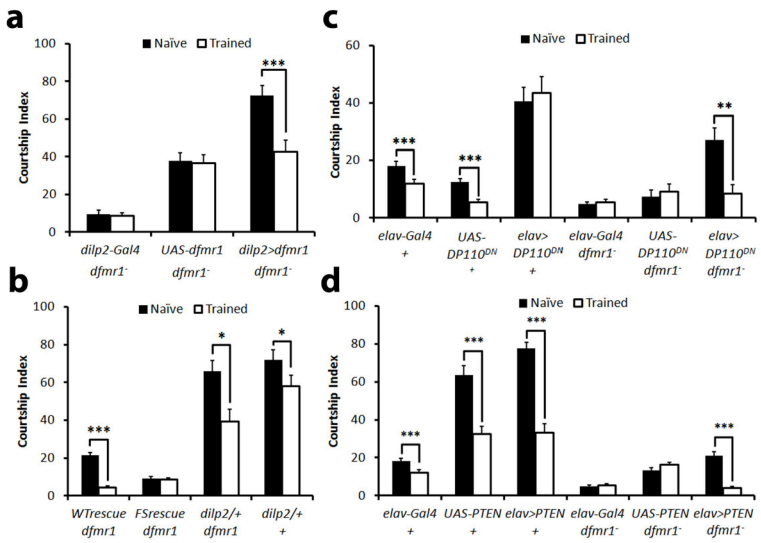


Figure 1| The short-term memory defect of *dfmr1* mutant flies is rescued by expression of *dfmr1* in the IPCs, genetic reduction of PI3K signaling activity. Graphs depict courtship indices of naïve males (black) and males trained with a mated female (white). **a,** Expression of *dfmr1* in the IPCs of *dfmr1* mutant flies restores memory, $p<0.001$. Memory is not seen in *dfmr1* mutants carrying the *dilp2-Gal4* or *UAS-dfmr1* transgenes alone. **b,** STM is restored in *dfmr1* mutants heterozygous for a *dilp2* mutation, $p<0.05$. **c,** STM is restored in *dfmr1* mutants expressing $DP110^{DN}$ pan-neuronally, $P<0.01$. **d,** STM is restored in *dfmr1* mutants over-expressing PTEN pan-neuronally, $P<0.001$. All error bars depict s.e.m.

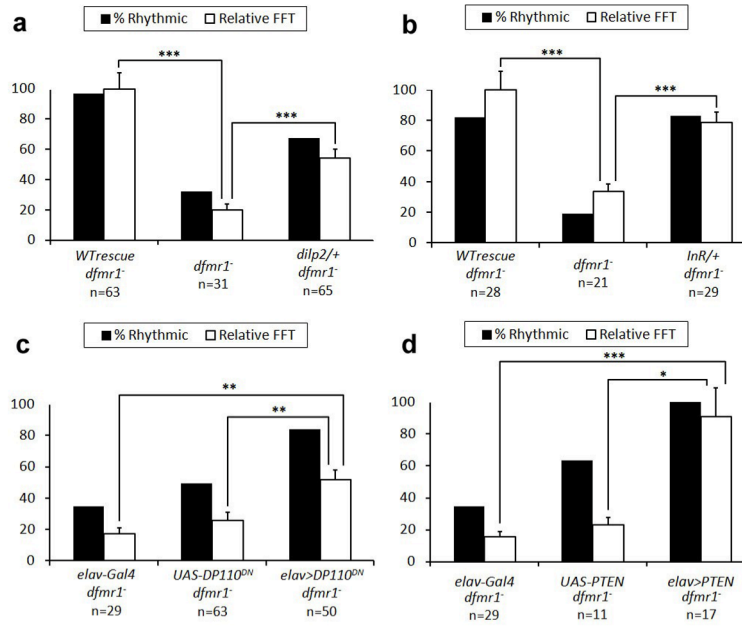


Figure 2| Genetic reduction of the insulin pathway rescues the circadian defect observed in *dfmr1* mutants. a-d, Panels show the percent rhythmic (black) and relative FFT values (white) for genetic combinations testing the effect of reducing insulin signaling in *dfmr1* mutants on circadian behavior. **a,** Circadian behavior of *dfmr1* mutants with the *WTrescue* transgene or with one copy of a null allele of *dilp2* (*dilp2*/+, *dfmr1*$^{-}$) display significantly improved circadian behavior relative to *dfmr1* mutants, p<0.001. **b,** Circadian behavior of *dfmr1* mutants with the *WTrescue* transgene or with one copy of a mutant allele of the insulin receptor (*InR*/+, *dfmr1*$^{-}$) display significantly improved circadian rhythmic strength relative to *dfmr1* mutants, p<0.001. **c,** Circadian behavior of *dfmr1* mutants with both *elav-Gal4* and *UAS-DP110*^{DN} (*elav>DP110*^{DN}*, dfmr1*$^{-}$) display significantly improved rhythmicity relative to *dfmr1* mutants with either transgene alone (*elav-Gal4, dfmr1*$^{-}$) and (*UAS-DP110*^{DN}*, dfmr1*$^{-}$), p<0.01. **d,** Circadian behavior of *dfmr1* mutants with both *elav-Gal4* and *UAS-PTEN* (*elav>PTEN, dfmr1*$^{-}$) display significantly improved circadian behavior relative to *dfmr1* mutants with either transgene alone (*elav-Gal4, dfmr1*$^{-}$) and (*UAS-PTEN, dfmr1*), p<0.001 and p<0.05 respectively. Significance denoted as described in Fig. 1. Error bars represent s.e.m.

Task 5: We have tested the effect of treating the *dfmr1* mutants with the Gsk-3beta inhibitor AR-A014418 during development and in adults. While we have not detected an effect on naïve courtship, we have found that treatment of *dfmr1* mutants during adulthood rescues the short-term memory deficit.

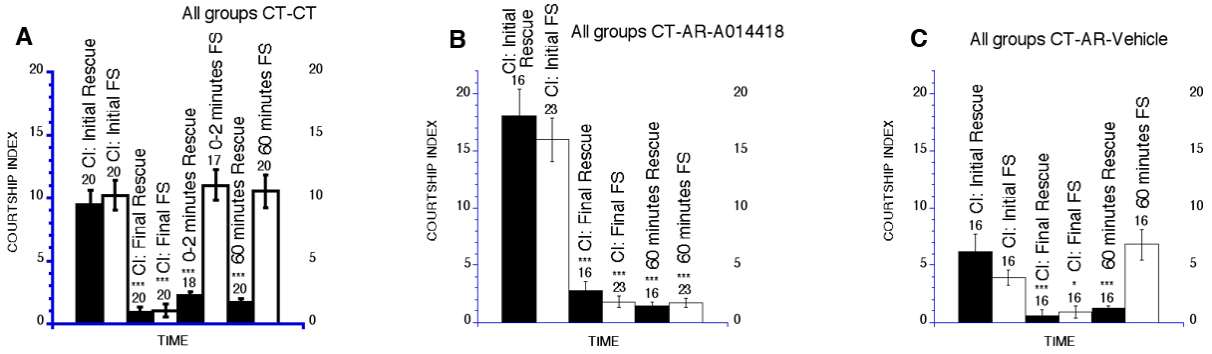
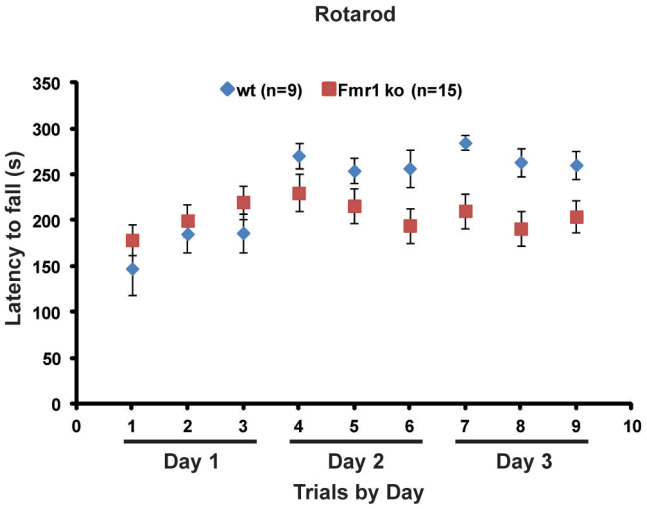
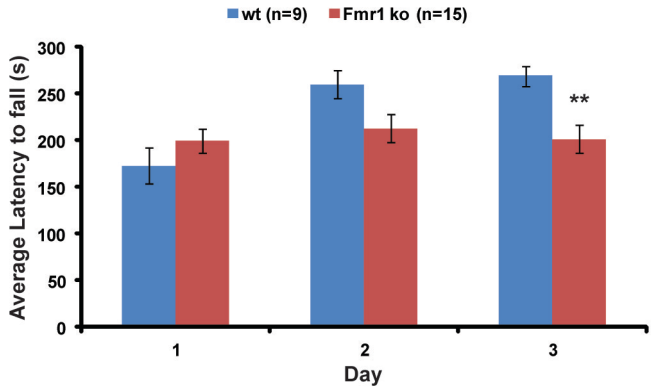


Figure 3. Treatment of the *dfmr1* mutants with the Gsk 3beta inhibitor AR-A014418 rescues short term memory. A) Both *dfmr1*mutants (FS) and controls (Rescue) display normal learning during training (Intial vs Final). The mutants however fail to display memory (reduced courtship) after the training. B) Treatment with AR-A014418 during adulthood rescues the short-term memory deficit (60 minutes) and has no negative effect on memory in the controls. C) Vehicle treatment for AR-A014418 has no effect on memory.

Task 12c. In testing the *dfmr1* mutants for behavioral phenotypes, we have found that few reported phenotypes could be reproduced in our hands using the Bl6 genetic background. We have therefore initiated testing in a 129XBl6 background and so far have found phenotypes to be more clear. We have now established conditions to perform the rotorod test that show a clear and reproducible difference between the mutants and littermate controls. We also have initial results that indicate the that object displacement assay is revealing a phenotype and the Y-maze is displaying a phenotype, however more n's need to be tested in these last two assays to determine if we can obtain statistically significant differences (not shown).





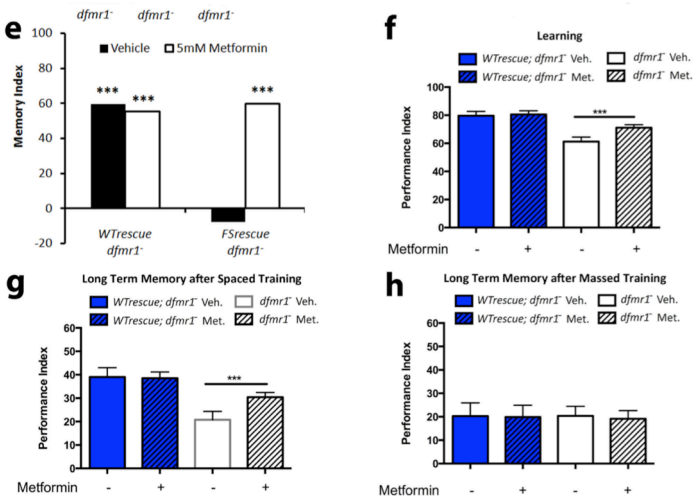


Figure 4| Treatment with metformin rescues memory in *dfmr1* mutants. Panels show the effect of metformin treatment on memory. **e,** STM is measured using memory index (MI) calculated from the courtship indices (CIs) of naïve (untrained) and trained flies using the formula $MI=(CI_{naive}-CI_{trained})/CI_{naive}$. Error bars are not shown for memory indices. **e,** *dfmr1* mutants treated with metformin demonstrated STM, p<.001 in contrast to *dfmr1* mutants treated with vehicle alone which did not demonstrate memory. N ranges from 36-86. **f-h,** Flies were also tested using the classical olfactory conditioning memory paradigm. Performance index (PI) represents the percent of flies which avoid the shock-conditioned odor. **f,** Immediate olfactory conditioning memory, named learning, was significantly improved in *dfmr1* mutants after being administered a dose of metformin (p <0.0001).No effect was observed in wild-type controls. N = 6 PIs per group. **g,** One day memory after spaced training is significantly improved in *dfmr1* mutant flies after being given a dose of metformin overnight before training. (p <0.00018) N = 8 PIs per group. **h,** One day memory after massed training did not differ between groups. N = 8 PIs per group. Graph depicts mean ± s.e.m.

Reportable outcomes:
Key Research Accomplishments:

Task 2a-The outcrossing of *Gsk-3beta*, *IPPase*, *InsP3R*, *Rheb*, *S6K* mutant stocks and the transgenic stocks *UAS-AMPK*, *UAS-4EBP* is complete we are now in the process of initiating the behavioral testing with these stocks.

Task 4. Test PI3K antagonists on the *Drosophila* fragile X model.
4a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone.
4b. Genetically validate the results obtained with the PI3K inhibitors.

Task 5. Test Gsk-3Beta antagonists on the *Drosophila* fragile X model.
5a. Test naïve courtship, learning during training (LDT), and memory (STM) in *dfmr1* mutant and control flies treated with drug or vehicle with continuous, development alone or adulthood alone.

8a. Examine naïve courtship, learning during training and memory in *dfmr1* mutants and controls treated with metformin vehicle during development alone, adulthood alone and during both times.

Ongoing tasks:

Task 1c. Perform biochemical analysis to determine effects of PDE-4 inhibition on PI3K and Akt activity and smRP6 levels.

Using an elisa assay to quantitate cAMP levels, we have now established that the *dfmr1* mutants have reduced resting levels of cAMP. We have also determined that treatment with rolipram can rescue the deficit of cAMP. Therefore we are in a position to now examine the effect of PDE-4 inhibition on PI3K, Akt and smRP6 levels.

2b. Molecular and genetic validation of genetic stocks. The *$InsP_3R$*, *dfmr1* and *Rheb*, *dfmr1* stocks may take additional time as the genes are genetically close to *dfmr1* on the 3rd chromosome.

5b. Genetically validate the results obtained with the Gsk-3Beta inhibitors.
5c. Perform biochemical analysis to determine effects of Gsk-3Beta inhibition on PI3K and Akt activity and smRP6 levels.

8a. Examine naïve courtship, learning during training and memory in *dfmr1* mutants and controls treated with AlCAR and vehicle during development alone, adulthood alone and during both times.

12c. Perform behavioral testing battery on *FMR1* KO and control mice.

13c. Perform behavioral testing on *FMR1* KO and control mice that are treated with PDE- 4, PDE-8 inhibitors or vehicle.

Manuscripts Published:

1) Wolman MA, de Groh ED, McBride SM, Jongens TA, Granato M, and Epstein JA. Modulation of cAMP and ras signaling pathways improves distinct behavioral deficits in a zebrafish model of neurofibromatosis type 1. Cell Rep. 2014 Sep 11;8(5):1265-70. doi: 10.1016/j.celrep.2014.07.054. Epub 2014 Aug 28.

2) PDE-4 inhibition rescues aberrant synaptic plasticity in Drosophila and mouse models of Fragile X syndrome.
Choi C.H., Schoenfeld B.P., Bell A.J., Hinchey J., Choi R.J., Hinchey P., Kollaros M., Gertner M.J., Ferrick N.J., Terlizzi A.M., Yang Y., Woo N.H., Tranfaglia M.R., Siegel S.J., McDonald T.V., Jongens T.A., McBride S.M.J.. J. of Neuroscience 2015 Jan 7;35(1):396-408. doi: 10.1523/JNEUROSCI.1356-12.2015.

3) Insulin signaling misregulation underlies circadian and cognitive deficits in a Drosophila fragile X model. Monyak RE, Emerson D, Schoenfeld BP, Zheng X, Chambers DB, Rosenfelt C, Langer S, Hinchey P, Choi CH, McDonald TV, Bolduc FV, Sehgal A, McBride SM, Jongens TA.
Mol Psychiatry. 2016 Apr 19. doi: 10.1038/mp.2016.51.

4) Multiple Drug Treatments That Increase cAMP Signaling Restore Long-Term Memory and Aberrant Signaling in Fragile X Syndrome Models. Choi CH, Schoenfeld BP, Bell AJ, Hinchey J, Rosenfelt C, Gertner MJ, Campbell SR, Emerson D, Hinchey P, Kollaros M, Ferrick NJ, Chambers DB, Langer S, Sust S, Malik A, Terlizzi AM, Liebelt DA, Ferreiro D, Sharma A, Koenigsberg E, Choi RJ, Louneva N, Arnold SE, Featherstone RE, Siegel SJ, Zukin RS, McDonald TV, Bolduc FV, Jongens TA, McBride SM. Front Behav Neurosci. 2016 Jun 30;10:136. doi: 10.3389/fnbeh.2016.00136. eCollection 2016.

Conclusions:

The overall objective of the work we have accomplished so far was to examine the efficacy of pharmacologically inhibiting PDE-4 activity to correct synaptic plasticity impairments in the fly and mouse models of Fragile X syndrome. The *Drosophila* Fragile X model recapitulates the most debilitating aspect of the disease in humans, namely impaired cognitive function. In our further dissection of the proteins involved in the mGluR signaling cascade, we identified PDE-4 as a potential substrate whose inhibition may be beneficial in restoring proper intracellular signaling in the Fragile X model (Fig. 1A). Based on the fly data, tissue culture work, the mouse model and samples from humans afflicted with Fragile X syndrome, we speculated that cAMP levels are suppressed (Berry-Kravis and Sklena, 1993; Berry-Kravis et al., 1995; Berry-Kravis and Ciurlionis, 1998; McBride et al., 2005; Kelley et al., 2007). PDE-4 inhibition should increase cAMP signaling by preventing the breakdown of cAMP that is produced during synaptic stimulation. Fragile X flies chronically treated in adulthood with PDE-4 inhibitors, or with genetically reduced levels of PDE-4, demonstrated intact immediate recall and short-term memory, validating PDE-4 inhibition as a potential novel therapeutic target for the treatment of synaptic plasticity impairments in Fragile X. We have validated the findings that indicate that cAMP levels are decreased in *dfmr1* mutants and that they are normalized by treatment with the drug rolipram. This finding adds to the growing body of literature demonstrating that pharmacologic treatment initiated in adulthood may have efficacy for the treatment of cognitive disorders that are already present in childhood as was first demonstrated in animal models of Fragile X and Neurofibromatosis type 1 in 2005 (Li et al., 2005; McBride et al., 2005; for review see Raymond and Tarpey, 2006; or Walsh et al., 2008).

In summary or work demonstrates that PDE-4 inhibition is a novel therapeutic target for the treatment of Fragile X. Prior to this work, it has only recently been demonstrated that enhanced LTD in the Fragile X model could be abrogated by chronic pharmacologic treatment (Choi et al., 2011). Equally as important is the demonstration that treatment in adulthood alone can rescue the phenotype, meaning that the phenotype is not irreversibly determined by pathogenic developmental circuitry. These findings urge the need for further exploration of PDE-4 inhibition as a potential therapy in Fragile X patients and in animal models of fragile X. Additionally, this work is a stepping stone for the field to begin a further pharmacologic dissection of the pathogenic signaling leading to aberrant LTD in the Fragile X model mouse, with the hope of these findings allowing the treatment of patients afflicted with Fragile X.

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